

Research article



Reduced expression of p27 is a novel mechanism of docetaxel resistance in breast cancer cells

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Abstract

Introduction Docetaxel is one of the most effective chemotherapeutic agents in the treatment of breast cancer. Breast cancers can have an inherent or acquired resistance to docetaxel but the causes of this resistance remain unclear. However, apoptosis and cell cycle regulation are key mechanisms by which most chemotherapeutic agents exert their cytotoxic effects.

Methods We created two docetaxel-resistant human breast cancer cell lines (MCF-7 and MDA-MB-231) and performed cDNA microarray analysis to identify candidate genes associated with docetaxel resistance. Gene expression changes were validated at the RNA and protein levels by reverse transcription PCR and western analysis, respectively.

Results Gene expression cDNA microarray analysis demonstrated reduced p27 expression in docetaxel-resistant breast cancer cells. Although p27 mRNA expression was found to be reduced only in MCF-7 docetaxel-resistant sublines (2.47-fold), reduced expression of p27 protein was noted in both MCF-7 and MDA-MB-231 docetaxel-resistant breast cancer cells (2.83-fold and 3.80-fold, respectively).

Conclusions This study demonstrates that reduced expression of p27 is associated with acquired resistance to docetaxel in breast cancer cells. An understanding of the genes that are involved in resistance to chemotherapy may allow further development in modulating drug resistance, and may permit selection of those patients who are most likely to benefit from such therapies.

Keywords: breast cancer, docetaxel, drug resistance, gene expression, p27

Introduction

Recent developments in chemotherapy have focused on the taxanes docetaxel and paclitaxel. Docetaxel is used in the treatment of breast cancer and is being evaluated in other solid tumours, including lung, gastro-oesophageal and, more recently, prostate cancers [1-3]. It is currently the most effective agent in the treatment of patients with advanced breast cancer. Up to 50% of patients who have previously been treated with or without anthracycline drugs, and have developed disease recurrence, will exhibit an objective response to docetaxel therapy [4]. Unfortunately, however, many patients do not respond to docetaxel or, having had an initial response, develop disease progression. This may occur either due to an inherent or an acquired resistance to docetaxel.

The mechanisms of docetaxel activity include binding to the β -tubulin subunits of microtubules, which prevents their depolymerization and thus blocks cell growth in the G₂-M phase. This consequently results in cell death by phosphorylation of bcl-2 [5], which is integral to the apoptotic pathway. However, the mechanisms of docetaxel resistance are poorly understood, although there are some that have been identified as probably being involved in resistance. For example, mutations in the β -tubulin gene [6] and differential expression of β -tubulin isotypes have been associated with resistance to docetaxel and paclitaxel in breast, ovarian and lung cancers [7-9]. Furthermore, in lung and prostate cancers docetaxel has been shown to induce expression of the p27 protein, which is another key protein involved in apoptosis [5,10].

bp = base pairs; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCR = polymerase chain reaction; TBST = Tris-buffered saline with 0.1% (vol:vol) Tween 20.

The *p27^{Kip1}* gene is a member of the cyclin-dependent kinase inhibitors, which arrest progression of the cell cycle [11]. The p27 protein interacts with cdk2 and cyclin E to prevent subsequent entry into S phase of the cell cycle [12-14]. It also acts as a tumour suppressor gene and has been shown to be involved in cell adhesion, apoptosis and triggering of differentiation [13,15-17]. Unlike many other tumour suppressor genes, however, gene mutation and loss of heterozygosity is infrequent, although *p27* levels may be controlled by DNA methylation [18] and protein degradation through the ubiquitin pathway [19].

The clinical significance of this protein is supported by the fact that loss of p27 expression has been shown to be a strong predictor of reduced survival in patients with breast cancer and also correlates with increasing histological grade. Furthermore, loss of p27 is involved in tumour resistance to hormonal treatment [20-23]. In experimental animal models, mice that lack p27 protein expression are larger than wild-type mice and develop large organs and pituitary tumours, most probably resulting from uncontrolled cell proliferation [24-26]. Overexpression of p27, by recombinant adenovirus, can also induce spontaneous apoptosis and cell cycle arrest in breast and oral carcinomas *in vitro* [27,28]. Other studies suggest that p27 expression can have an antiapoptotic effect and prevent drug-induced apoptosis by DNA damaging agents, such as cisplatin, leading to drug resistance [29]. The effect of p27, therefore, may be cell or tissue specific [14,30]. The ability of p27 to initiate apoptosis may account for its possible involvement in chemotherapy-induced apoptosis [31].

Whether modulation of p27 expression plays a role in the development of resistance to docetaxel in breast cancer cells has not previously been investigated. This study focuses on the role played by p27 in docetaxel resistance, at RNA and protein levels, in an *in vitro* breast cancer model of acquired docetaxel resistance. In addition, our use of cDNA microarrays allowed identification of other candidate genes, which may be important for subsequent evaluation of docetaxel resistance.

Methods

Cell culture

Human breast cancer cell lines MCF-7 (oestrogen receptor positive) and MDA-MB-231 (oestrogen receptor negative) were cultured in RPMI-1640 medium, supplemented with 10% (vol:vol) foetal calf serum, 0.2% (weight:vol) sodium bicarbonate and 1% (vol:vol) penicillin-streptomycin, at 37°C in a humidified atmosphere containing 5% carbon dioxide. The cells were made resistant to docetaxel by short-term *in vitro* exposure to docetaxel (a gift from Aventis Pharma Ltd, West Malling, Kent, UK) for 1 hour, which was immediately followed by washing of the cells several times with culture media, trypsinization, and splitting the cells for

subsequent cell growth recovery. The cells were initially exposed to 10 nmol/l docetaxel increasing to 500 nmol/l for 1 hour. After this point, the cells were exposed to 1 µmol/l docetaxel increasing to 30 µmol/l docetaxel for 24 hours.

Cytotoxic assay

Docetaxel resistance was demonstrated in cell lines by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay [32]. The MCF-7 and MDA-MB-231 cells and their resistant sublines were plated onto 96-well plates, with a seeding density of 5×10^4 cells/well in 100 µl RPMI-1640 culture medium. Cells were exposed to varying concentrations of docetaxel and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 24 hours. Following exposure of cells to docetaxel, cells were treated with 50 µl 12 mmol/l MTT dye and incubated at 37°C for 4 hours. Following this incubation period, all the liquid was aspirated from each well before the addition of 200 µl dimethylsulphoxide in order to dissolve the MTT-formazan crystals. The number of viable cells was determined by measuring the absorbance at 570 nm and 630 nm for each well using a microplate spectrophotometer (DynaTech MR5000, DynaTech Laboratories Inc., Chantilly, VA, USA). All experiments were repeated three times with six replicates per experiment.

The concentration of docetaxel required to cause 50% inhibition of cell growth was calculated by direct cell counts in the presence of docetaxel. MCF-7 and MDA-MB-231 cells and their resistant sublines were plated onto petri dishes in RPMI-1640 culture medium. Cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 24 hours. After this time, the medium was removed and replaced with fresh culture medium containing varying concentrations of docetaxel and incubated for a further 24 hours. The medium was then removed and the cells resuspended in 1 ml culture medium. In order to count the cells, 100 µl of the cell solution was added to 100 µl trypan blue, and the cells were counted using a haemocytometer by light microscopy. The relative survival was expressed as a percentage inhibition of growth relative to the control. All experiments were repeated two times with two replicates per experiment.

cDNA microarray analysis

Total RNA was isolated from the cell lines using Trizol reagent (Invitrogen, Paisley, UK) in accordance with the manufacturer's instructions. RNA quality was enhanced using the RNeasy Mini Kit (Qiagen, Crawley, West Sussex, UK), and the integrity of RNA was checked by separating RNA by electrophoresis through a 1% (weight:vol) agarose gel. Following electrophoresis, the presence of two distinct bands (representing the 28S and 18S species of rRNA), with no or minimal smearing, confirmed the presence of

undegraded RNA. In addition, the purity of RNA was determined by measuring the absorbance at 260 nm and 280 nm using spectrophotometry. Expression of cancer pathway genes was evaluated using Cancer Pathway Finder gene expression arrays (Super Array Inc., Frederick, MA, USA), in accordance with the manufacturer's instructions. In brief, gene-specific biotin-labelled cDNA probes were generated from 2.5 µg total RNA using gene-specific primers for reverse transcription using 200 U MMLV reverse transcriptase (Promega, Southampton, UK) and 40 U RNase inhibitor (Promega). The cDNA probe was denatured by heating at 94°C for 5 min and quickly cooled on ice before hybridization, with the cDNA membrane array, at 68°C overnight. Prior to hybridization, the membrane was prehybridized at 68°C for 30 min in the supplied hybridization buffer (Super Array Inc.). The membrane was washed accordingly and then the hybridization signals were detected using the CPD Star chemiluminescent detection kit (Super Array Inc.). Following this, the membrane was scanned with a Fluor S phosphorimager (Biorad, Hemel Hempstead, Hertfordshire, UK) and the scanned image was converted into digital data and analyzed using GEArray Analyzer software (Super Array Inc.)

Reverse-transcription polymerase chain reaction

Total RNA (2 µg) was reverse transcribed in 20 µl reverse transcription reaction buffer (50 mmol/l Tris, pH 8.3, 75 mmol/l potassium chloride, 15 mmol/l magnesium chloride, 10 mmol/l dithiothreitol), containing 250 ng random hexamers (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK), 500 µmol/l dNTP and 200 U Superscript II reverse transcriptase (Invitrogen). Reverse transcription was performed at 42°C for 1 hour, followed by heating at 70°C for 10 min. The cDNA (1 µl) was amplified in 25 µl reaction volumes, which contained the following components: 10 mmol/l Tris, pH 8.3, 50 mmol/l potassium chloride, 0.1 mg/ml gelatin, 2.5 mmol/l magnesium chloride, 200 µmol/l dNTP, 10 pmol of each oligonucleotide primer, and 0.5 U Taq DNA polymerase (Roche Diagnostics, Lewes, East Sussex, UK). PCR was carried out under the following conditions: 94°C for 2 min followed by 23 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension step of 72°C for 10 min. The oligonucleotide primer sequences for p27 produced a 253 bp PCR product: sense primer 5'-TGG AGA AGC ACT GCA GAG AC-3' and antisense primer 5'-GCG TGT CCT CAG AG T TAG CC-3'. In addition, QuantumRNA™ 18S internal control standards (Ambion Inc., Huntingdon, UK), which produced a PCR product of 489 bp, were used at a ratio of 10:1 competitors to primers, in accordance with the manufacturer's instructions, to act as an internal loading control to normalize between samples during densitometry. PCR products were electrophoresed through a 1.5% (weight:vol) agarose gel. Gel images were captured by Genesnap software (Syngene, Cambridge, UK) and band

densities calculated using a Fluor S phosphorimager (Biorad). The experiments were repeated in triplicate with RNA isolated from two independent extractions.

Western analysis

Cells were homogenized in a lysis buffer (20 mmol/l Tris, 0.25 mol/l sucrose, 10 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 25 mmol/l sodium β-glycerophosphate, 50 mmol/l sodium fluoride, pH 7.5). Prior to use, 0.1% (vol:vol) protease inhibitor cocktail (Sigma, Gillingham, Dorset, UK) was added. Cells were lysed by sonication and aspiration through a 25 G needle. Protein concentration was quantified using the DC Protein Assay (Biorad). Ten micrograms of protein was electrophoresed through a precast 15% polyacrylamide gel (Cambrex Bioscience, Nottingham, UK) for 2 hours at 25 V. Following electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Biorad) and each membrane was blocked with 5% (weight:vol) skimmed milk in Tris-buffered saline with 0.1% (vol:vol) Tween 20 (TBST) at 4°C overnight. Each membrane was incubated with 1:250 dilution (in 5% [weight:vol] milk/TBST solution) of monoclonal mouse anti-human p27 antibody (DakoCytomation, Ely, UK) or 1:5000 β-actin (Abchem, Cambridge, UK) for 1 hour at room temperature. β-Actin was used as an internal loading control to normalize between samples during densitometry. The membrane was washed six times for 5 min in TBST solution before addition of the secondary antibody, HRP labelled goat anti-mouse IgG (Oncogene Research Products, San Diego, CA, USA), at a 1:5000 dilution in 5% (weight:vol) milk/TBST solution for 1 hour at room temperature. The membrane was further washed five times for 5 min in TBST solution and then once for 5 min in phosphate-buffered saline solution. Bands were visualized using ECL+plus™ chemiluminescent detection kit (Amersham Pharmacia) in accordance with the manufacturer's instructions, and the blots were scanned with a Fluor S phosphorimager (Biorad). The experiments were repeated in duplicate with protein isolated from two independent extractions. Cells were also checked by flow cytometry to ensure they were in the same cell cycle phase (data not shown).

Results

Docetaxel-induced cytotoxicity is reduced in docetaxel-resistant sublines

First, the *in vitro* effect of docetaxel on cell growth was determined using a standard cell viability assay. This demonstrated that docetaxel was more cytotoxic in MCF-7 and MDA-MB-231 cells than in their resistant sublines, which were able to withstand 24 hours of exposure of 30 µmol/l docetaxel. MCF-7 docetaxel-resistant sublines (MCF-7 TAX30) exhibited a 666-fold greater resistance to docetaxel than did MCF-7 cells. Furthermore, MDA-MB-231 docetaxel-resistant sublines (MDA-MB-231 TAX30)

Table 1**50% Inhibitory concentrations for docetaxel in breast cancer cell lines**

Cell line	IC ₅₀ docetaxel (nmol/l)
MCF-7	15
MCF-7 TAX30	10000
MDA-MB-231	40
MDA-MB-231 TAX30	55000

MCF-7 TAX30 and MDA-MB-231 TAX30 are docetaxel-resistant sublines. IC₅₀, 50% inhibitory concentration.

exhibited a 1375-fold greater resistance to MDA-MB-231 cells (Table 1).

Membrane-based microarray identifies p27 as a candidate gene associated with docetaxel resistance

In order to identify genes associated with docetaxel resistance, we applied a cDNA expression array to our *in vitro* docetaxel-resistant breast cancer model. The human cancer pathway array contained 96 genes that are involved in various pathways of cancer development, including tumour suppressor genes, oncogenes, signal transduction pathway genes, growth factors and receptors, and genes involved in angiogenesis. There was a 2.3-fold downregulation of p27 in MCF-7 docetaxel-resistant cells as compared with the parental cells (Table 2). Because p27 is a direct target for docetaxel, we selected this as a candidate for the present study. In addition, there were further changes in gene expression as illustrated in Table 2.

Expression of p27 mRNA is different in two docetaxel-resistant breast cancer cell lines

In order to confirm the results obtained using cDNA microarrays, the expression of p27 at the RNA level was determined by semiquantitative reverse transcription PCR analysis. In comparison with MCF-7 cells, MCF-7 docetaxel-resistant sublines exhibited a 2.47 ± 0.15 fold decrease in p27 mRNA expression (Fig. 1). In contrast, p27 mRNA expression was increased in MDA-MB-231 resistant cells (2.29 ± 0.76 fold).

Reduction in p27 protein in docetaxel-resistant breast cancer cells

The conflicting p27 mRNA expression difference between the two docetaxel-resistant breast cancer cell lines was validated at the protein level by western analysis. It was observed that p27 protein expression was reduced in both docetaxel-resistant cell lines (Fig. 2). There was a 2.83 ± 0.15 fold decrease in MCF-7 resistant cells and a 3.80-fold decrease in MDA-MB-231 resistant cells.

Discussion

This study is the first to demonstrate that reduced p27 expression is associated with acquired resistance to docetaxel in breast cancer cells *in vitro*. There may be several mechanisms that are involved in this process and that may be the result of either transcriptional or translational regulation, with modulation of gene expression and/or resultant production of the protein. In addition, there may be an increased degradation of p27 via the ubiquitin degradation pathway.

Previous studies have indicated that decreased expression of p27 is associated with drug resistance to hormonal therapy (e.g. tamoxifen) in breast cancer cells and to chemotherapy (e.g. cisplatin in malignant gliomas), mediated through mitogen-activated protein kinase activation [20,21,33]. It has also been reported that reduced p27 protein expression in epithelial ovarian tumours was observed, as compared with normal ovaries, from patients receiving cisplatin and paclitaxel treatment [34]. Reduced p27 expression was significantly associated with presumed chemoresistance (i.e. the patients had persistent disease after treatment) [34]. Furthermore, resistance to the anti-tumour agent rapamycin (a potent immunosuppressant that was recently reported to be effective in breast cancer treatment) has also been shown to be associated with reduced p27 expression in murine myogenic cells [35]. Another study noted that decreased p27 expression correlated with chronic *Helicobacter pylori* infection, which resulted in a phenotype that was resistant to apoptosis (and therefore potentially drug resistant because the majority of chemotherapeutic agents induce apoptosis) in gastric cancer patients [36]. It is possible that decreased p27 expression may prevent apoptosis or arrest cells in G₁ phase, thereby reducing the cytotoxic effect of chemotherapeutic drugs that act on proliferating cells.

Increased p27 expression has also been implicated in causing drug resistance in leukaemia cell suspensions by preventing apoptosis [30]. The authors of that study reported that overexpression of p27 in the cells resulted in resistance to drug-induced apoptosis, including DNA damaging and non-DNA damaging agents. In addition, increased p27 expression is associated with cisplatin resistance in colorectal cancer cells [29]. Naumann and colleagues [33] also reported that increased p27 expression could increase resistance to vincristine, camptothecin and teniposide in malignant glioma cells, whereas reducing p27 expression (by antisense mRNA) sensitized the same cells to cisplatin, although effects appeared to be drug specific. Therefore, it may be that the effects of an individual antitumour agent are dependent on the mechanisms of action involved.

Table 2**Gene expression differences in docetaxel-resistant breast cancer cells**

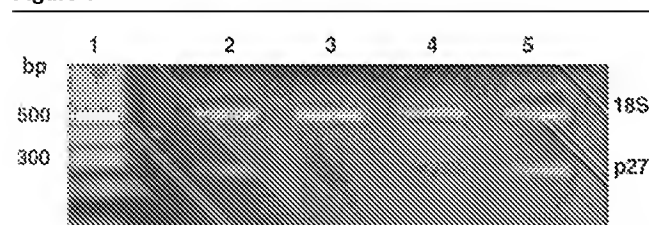
Gene name	Gene symbol	Chromosome	UniGene	Gene expression ¹
HER2	<i>ErbB2</i>	17q11.2-q12	Hs. 446352	+11.4
IL-8	<i>IL8</i>	4q13-q21	Hs. 624	+8.2
FGF2	<i>FGF2</i>	4q26-q27	Hs. 284244	+6.2
Survivin	<i>BIRC5</i>	17q25	Hs. 1578	+3.7
p38 MAPK	<i>MAPK14</i>	6p21.2-p21.31	Hs. 79107	+3.56
TNFR1	<i>TNFRSF1A</i>	12p13.2	Hs. 159	-7.5
Bax	<i>BAX</i>	19q13.3-q13.4	Hs. 159428	-5.1
Cyclin E1	<i>CCNE1</i>	19q12	Hs. 244723	-2.8
Killer/DR5/TRAILR2	<i>TNFRSF10B</i>	8p21-p22	Hs. 51233	-2.76
p27Kip1	<i>CDKN1B</i>	12p12-p13.1	Hs. 238990	-2.3

¹Ratio of gene expression between resistant sublines and parental cell lines: '+' indicates an increase in gene expression in the resistant sublines, and '-' indicates a decrease in gene expression in the resistant sublines.

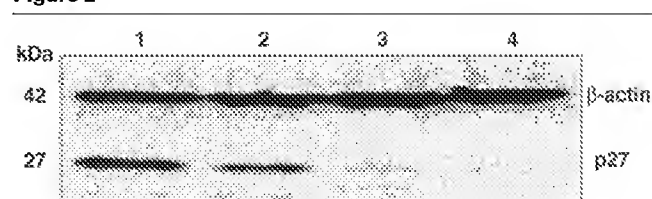
It has generally been regarded that increased p27 can initiate apoptosis by increasing the expression of the proapoptotic protein bax [31]. Docetaxel-induced cytotoxicity is mediated by increased p27 expression in lung and prostate cancers [10]. Furthermore, if the expression of p27 is increased, by using the proteasome inhibitor PS-341 (which inhibits the activity of the proteasome enzyme) [37], then this increases docetaxel-induced apoptosis in lung cancer cells [38]. Reduced p27 levels, therefore, may prevent cell death occurring by this mechanism. Indeed, p27 was demonstrated to induce apoptosis in response to low doses of docetaxel independent of bcl-2 phosphorylation, which is a recognized event for apoptosis regulation by docetaxel [5].

The precise mechanism by which decreased p27 expression may lead to docetaxel resistance is not yet fully under-

stood. The p27 protein is primarily involved in cell cycle arrest (G₁) via its ability to bind with and inactivate cyclin E-cdk2 complexes [39]. It is likely, therefore, that reduced p27 expression will prevent cell cycle arrest. In addition, downregulation of p27, with antisense molecules, leads to re-entry into the cell cycle, which leads to cell proliferation [40]. It has also been suggested that a loss of p27 may desensitize tumour cells to antimitogenic signals, thus preventing apoptosis in their evolution [41]. Continued exposure to docetaxel may therefore select for apoptosis-resistant tumour cells that express decreased levels of p27. One recent study demonstrated that a decrease in p27 expression in normal mammary tissue during pregnancy results in both an increase in cell proliferation and in apoptosis, suggesting that under normal circumstances a reduced expression of p27 would not result in a significant growth advantage [42]. If the apoptotic pathway is impaired, however, then cells could proliferate after exposure to docetaxel, and this may contribute to drug resistance.

Figure 1

Modulation of p27 mRNA expression in docetaxel-resistant breast cancer cells. Total RNA extracted from each cell line was amplified using specific oligonucleotide primer sequences and separated by agarose gel electrophoresis. The p27 and 18S internal control amplified PCR products produced fragment sizes of 253 bp and 489 bp, respectively. Lane 1, DNA size standards; lane 2, MCF-7 docetaxel-resistant subline; lane 3, MCF-7 breast cancer cells; lane 4, MDA-MB-231 breast cancer cells; lane 5, MDA-MB-231 docetaxel-resistant subline.

Figure 2

Modulation of p27 protein expression in docetaxel-resistant breast cancer cells. Protein extracted from each cell line was separated by PAGE, transferred to nitrocellulose membranes and probed with anti-human p27 and β -actin antibodies. Lane 1, MCF-7 breast cancer cells; lane 2, MCF-7 docetaxel-resistant subline; lane 3, MDA-MB-231 breast cancer cells; lane 4, MDA-MB-231 docetaxel-resistant subline.

In addition to p27, cDNA microarray analysis revealed several other genes with expression that may be altered in docetaxel-resistant breast cancer cell lines (Table 2). These include decreased expression of cell cycle (*CCNE1* and *CDKN1B*) and proapoptotic genes (*BAX* and *TNFRSF1A*), and increased expression of antiapoptotic genes (*BIRC5*), which could lead to resistance to the mechanisms of action of docetaxel. Increased expression of angiogenic (*IL8*) and growth factors (*ErbB2*) could also lead to a more aggressive cell type that is perhaps able to escape the cytotoxic effects of the drug. These changes, however, remain to be validated at the mRNA and protein levels.

One of the limitations of the present study is the use of an *in vitro* model of drug resistance, which is, by definition, an artificial environment. In particular, our experimental approach selected cells with an acquired resistance to docetaxel rather than utilizing cells that exhibit *de novo* resistance to the drug. We acknowledge that it is possible that different mechanisms may be involved in *de novo* docetaxel resistance. However, a previous study successfully used colon cancer cell lines and their resistant sublines to identify a candidate gene, thymidylate synthase, that is involved in resistance to thymidylate synthase inhibitors [43].

Conclusion

The results from this study used cDNA microarrays based on specific molecular pathways that are believed to be involved in tumour progression and drug resistance. There are other, more comprehensive cDNA microarray methods available, which produce copious amounts of data. However, our experimental approach provides a quicker, less complicated and more focused way to identify possible candidate genes. To the best of our knowledge, this is the first description of an *in vitro* model of acquired docetaxel resistance in breast cancer cells. We directly compared breast cancer cells with their docetaxel-resistant sublines to identify gene expression changes associated with docetaxel resistance. In particular, the fact that p27 protein expression is reduced in both docetaxel-resistant sublines suggests that it plays a role in docetaxel resistance in breast cancer. Further studies, involving genetic profiling, may be important in allowing identification of those patients who are most likely to respond to a particular treatment.

Competing interests

None declared.

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